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Identification of the amino acids comprising a surface-exposed epitope within the nucleotide-binding domain of the Na^+,K^+-ATPase using a random peptide library

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Abstract

Monoclonal antibodies that bind native protein can generate considerable information about structure/function relationships, but identification of their epitopes can be problematic. Previously, monoclonal antibody M8-P1-A3 has been shown to bind to the catalytic (α) subunit of the Na^+,K^+-ATPase holoenzyme and the synthetic peptide sequence 496-HLLVMK*GAPER-506, which includes Lys 501 (K*), the major site for fluorescein-5′-isothiocyanate labeling of the Na^+,K^+-ATPase. This sequence region of α is proposed to comprise a portion of the enzyme's ATP binding domain (Taylor, W.R. & Green, N.W., 1989, *Eur. J. Biochem. 179*, 241–248). In this study we have determined M8-P1-A3's ability to recognize the α-subunit or homologous E_1E_2-ATPase proteins from different species and tissues in order to deduce the antibody's epitope. In addition the bacteriophage random peptide or "epitope" library, recently developed by Scott and Smith (1990, *Science 249*, 386–390) and Devlin et al. (Devlin, J.J., Panganiban, L.C., & Devlin, P.E., 1990, *Science 249*, 404–406), has served as a convenient technique to confirm the species-specificity mapping data and to determine the exact amino acid requirements for antibody binding. The M8-P1-A3 epitope was found to consist of the five amino acid 494-PRHLL-498 sequence stretch of α, with residues PR*x*L*x* being critical for antibody recognition.

Keywords: bacteriophage "random peptide" library; epitope; monoclonal antibody; Na^+,K^+-ATPase

The sodium, potassium ATPase (EC 3.6.1.37, Na^+,K^+-ATPase), first identified by J.C. Skou in 1957, is a membrane-embedded enzyme that functions to maintain the sodium and potassium levels in all animal cells. As a consequence, it modulates such specialized functions as cell volume regulation, nutrient uptake, and the electromotive potential of excitable cells. The complete amino acid sequences for both its catalytic (α, 112 kDa) and glycoprotein (β, 35 kDa) subunits from a variety of species and tissues have been deduced from their cDNA sequences (see review, Lingrel et al., 1990). The Na^+,K^+-ATPase clearly belongs to a class of E_1E_2 pumps, which have a high degree of sequence identity and undergo very similar processes during catalytic turnover. They are all membrane-bound cation pumps that form phosphoenzyme intermediates during the reaction process (see review, Jorgensen, 1988). In addition, they are all similarly labeled by the fluorescent probe, fluorescein-5′-isothiocyanate (FITC) at the analogous Lys 501 on the catalytic subunit (Farley et al., 1984; Kirley et al., 1984). Covalent labeling at this site inactivates the enzyme primarily by blocking the binding of ATP. Consistent with the hypothesis that the covalently bound FITC resides in the ATP-binding site, ATP prevents FITC labeling and protects the enzyme against inactivation by FITC. Despite its inactivation of the enzyme's ATPase activity, FITC has proven to be an extremely useful probe because it undergoes a series of ligand-dependent fluorescent intensity changes that appear to correlate well with the conformational changes that the enzyme undergoes during its catalytic cycle (Karlish, 1980; Hegevary & Jorgensen, 1981). The fact that Na^+, K^+, Mg^{2+}, Pi, and ouabain all induce fluorescence intensity changes in enzyme-bound FITC suggests that most of the enzyme's functional parameters are intact even with the covalently bound probe. In addition to serving as the site for FITC linking, the sequence region of α that encompasses the Lys 501 (HLLVMKGAPER) has been the subject of considerable interest because it is highly conserved

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among E_1E_2-ATPases and is believed to comprise an initial portion of the putative nucleotide-binding domain. Analysis of the predicted secondary structures of these ATPases suggests a common folding pattern for this domain that is largely a repeated β-sheet/turn/α-helix pattern. Taylor and Green (1989) and Green and Stokes (1992) have developed a detailed model which suggests that the sequences immediately before Lys 501 make up one of the inaccessible β-sheets that help generate the nucleotide-binding cleft.

Previous studies in our laboratory have shown that monoclonal antibody M8-P1-A3, which was raised to the purified lamb kidney Na^+,K^+-ATPase, binds to the synthetic peptide HLLVMKGAPER-BSA conjugate (Ball & Loftice, 1987; Ball & Friedman, 1987). However, despite the fact that this antibody binds to a site that may form a portion of the ATP-binding site, it has been found not to affect enzyme activity. This contrasts with the report that a polyclonal antibody population raised to the synthetic peptide sequence -GAPER contained within the larger peptide partially inhibits activity (Xu & Kyte, 1989). In order to better understand the enzyme's structure and locate the binding site of M8-P1-A3, we have employed two different techniques to identify the exact amino acid sequences to which this antibody binds. Species-specificity mapping and a filamentous bacteriophage library of 15 amino acid random sequence inserts have enabled us to determine that the antibody-reactive synthetic peptide contains only a portion of the antibody's epitope. The monoclonal antibody's epitope has been found to consist of the sequences 494-PRHLL-498, a site adjacent but distinct from that of the GAPER sequence-directed antibodies.

Results and discussion

Epitope identification by species-specificity mapping

In previous studies we reported the observation that the α-subunit-directed antibody M8-P1-A3, with a 12-fold lower affinity, also bound to the synthetic peptide 496-HLLVMKGAPER-506 as coupled to bovine serum albumin (BSA) (Ball & Loftice, 1987). In additional competition binding studies, M8-P1-A3 was also found to bind weakly to the free peptide but not to the peptide–KLH conjugate. Thus, although the apparent binding site of M8-P1-A3 was identified, the specific amino acids comprising its epitope were not known, nor was it understood why M8-P1-A3 did not bind to the peptide–KLH conjugate. Furthermore, because this particular sequence stretch of the α1 isoform of the lamb kidney Na^+,K^+-ATPase is highly conserved in all three α isoforms (designated α1, α2, and α3) among different animal species, as well as other E_1E_2-ATPases (Table 1), it was expected that this antibody would have a broad range in its binding specificity. However, we found that M8-P1-A3 did not bind to either the rat kidney Na^+,K^+-ATPase nor to pig gastric H^+,K^+-ATPase when these proteins were adsorbed to microtiter plates for ELISA or nitrocellulose sheets for dot blot assays. The M8-P1-A3 did recognize these two proteins in Western blot analysis (Ball & Lane, 1986; Lane et al.,

Table 1. *Comparison of the amino acid sequences of E_1E_2 pumps in the region of the M8-P1-A3 epitope*[a]

E_1E_2 pumps		M8-P1-A3 affinity	Sequence
Na^+,K^+-ATPase			
Sheep	α1	High	^{489}ANAGE **PRHLL** VMKGAPER^{506}
Porcine	α1	High	PNTAE **PRHLL** VMKGAPER
Rat	α1	High	PNASE **PKHLL** VMKGAPER
Rat	α2	None	EDSPQ **S_HVK** VMKGAPER
Rat	α3	None	EDPND **NRYLL** VMKGAPER
Chicken	α1	Low	ANAGE **SRHLL** VMKGAPER
Xenopus	α	None	ANPSE **SRYIL** VMKGAPER
Torpedo electroplax	α	Low	ADKAD **SRYLL** VMKGAPER
E_1E_2-ATPase			
Pig gastric	H/K	High	EDPRD **PRHL_** VMKGAPER
Rabbit skeletal SR	Ca/Mg	None	SRAAV **GNKMF** V_KGAPEG
Synthetic peptide		Low	^{496}**HLL** VMKGAPER

[a] Western blot binding results demonstrated that M8-P1-A3 showed strong binding to the Na^+,K^+-ATPase α1-subunit from lamb, dog, and pig kidney and the pig gastric H^+,K^+-ATPase. It showed lower affinity binding to the rat α1, chicken α1 and *Torpedo* electroplax organ enzymes and no binding to rat α2 or α3 isoforms, *Xenopus* kidney or the rabbit skeletal sarcoplasmic reticulum (SR) Ca^{2+},Mg^{2+}-ATPase. Comparison of the sequences of these proteins suggested that the epitope should be limited to the sequence 494-EPRHLL. The amino acid sequences presented were deduced from the nucleotide sequencing of cloned cDNAs, they were compiled for the Na^+,K^+-ATPases by Lingrel et al. (1990), Ca^{2+},Mg^{2+}-ATPase (Maruyama et al., 1989), and the pig gastric H^+,K^+-ATPase (Maeda et al., 1988), and they are represented using the standard one letter–one amino acid code.

1986). These results suggested that both the tertiary structure as well as the linear amino acid sequences of the protein were important for antibody binding, and this antibody was capable of discriminating between different native and denatured forms of α.

Therefore, we undertook the screening of the antibody's ability to recognize the Na^+ pump from a variety of species as a means of deducing its site of binding using Western-blotted proteins so that protein sequences rather than conformations should play the major role in determining antibody affinity. As shown in Figure 1, a series of acrylamide gel-resolved purified enzyme and microsomal samples were matched by Coomassie blue staining for their levels of E_1E_2 α-subunit present and were also transferred to nitrocellulose sheets for Western blot analysis. Among these samples, M8-P1-A3 was found to bind strongly to the purified lamb, dog, pig, and rat kidney enzymes. It did not bind to microsomal samples of *Xenopus* kidney, and it had a low affinity for the α-subunits of the chicken kidney and *Torpedo* electroplax organ Na^+,K^+-ATPases. In addition, it recognized both pig and rat (not shown) gastric H^+,K^+-ATPases, but not rabbit skeletal sarcoplasmic reticulum (SR) Ca^{2+}-ATPase. As controls, all of these samples were recognized by the anti-α1 polyclonal serum 37292, and lamb Na^+,K^+-ATPase α1-directed monoclonal antibody, M12-P4-E9, reacted with all sam-

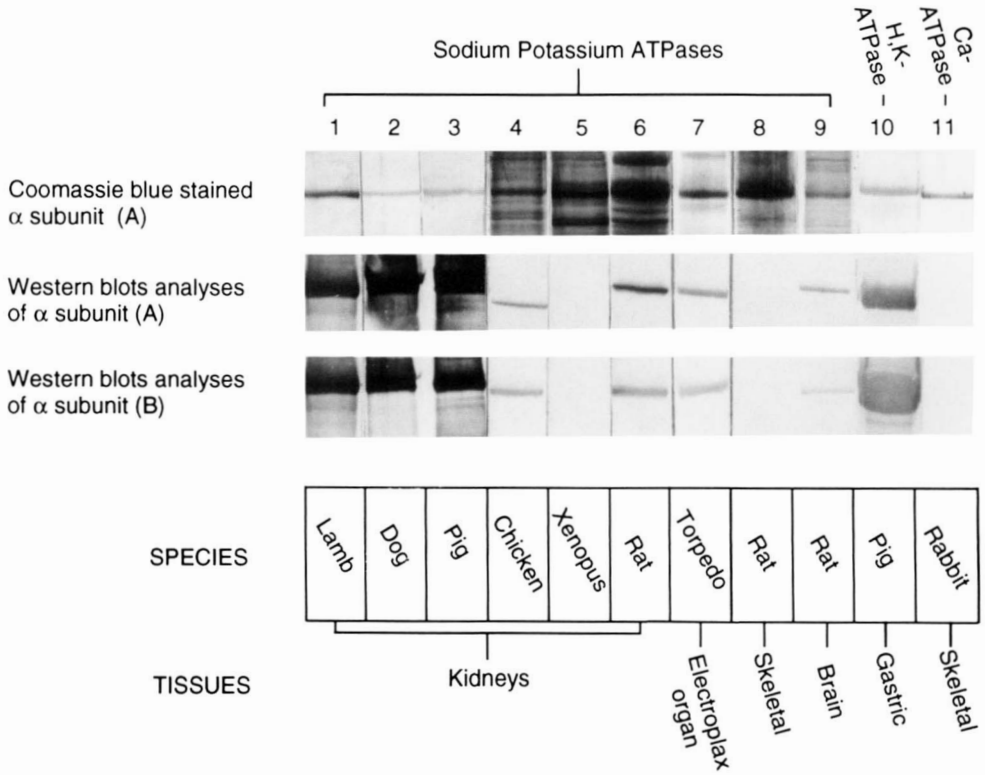

Fig. 1. Determination of the binding specificity of monoclonal antibody M8-P1-A3. Western blot analysis of antibody M8-P1-A3 binding to Na^+,K^+-ATPase α-subunits and E_1E_2-ATPases from different animal species and tissues was accomplished following SDS-PAGE resolution of the proteins in 10% acrylamide gels (7.5% for the rat kidney enzyme) and their electrophoretic transfer to nitrocellulose sheets. An approximate quantitation of the catalytic subunit present in each sample was accomplished by Coomassie blue staining of gel-resolved proteins. The nitrocellulose sheets were exposed to 100 nM M8-P1-A3, followed by the secondary antibody, rabbit anti-mouse IgG, $F(ab)'_2$ conjugated to horseradish peroxidase to detect antigen-bound antibody. A goat anti-rabbit IgG, $F(ab)'_2$ peroxidase conjugate secondary antibody was used to detect bound rabbit serum 37292. **A:** The top row (A) shows the Coomassie blue staining patterns for the catalytic subunit present in the various enzyme or microsomal samples that generate the Western blot staining pattern shown below in row 2 (A). The protein concentrations used were: purified lamb kidney Na^+,K^+-ATPase, 0.2 μg/well; pig kidney Na^+,K^+-ATPase, 1 μg; dog kidney Na^+,K^+-ATPase, 1 μg; rat kidney Na^+,K^+-ATPase, 3 μg; chicken kidney microsomes, 20 μg; *Xenopus* kidney microsomes and *Torpedo* electroplax organ, 10 μg; rat brain microsome samples 10 μg; pig gastric H^+,K^+- ATPase, 5 μg and the rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase, 1 μg. All samples contained levels of the α-subunit at least equivalent to that of 0.2 μg/well of the lamb kidney enzyme. **B:** The bottom row (B) shows the Western blot analysis of M8-P1-A3 binding to samples at the same or higher concentrations from those shown in A, to confirm or clarify those results. Purified lamb kidney Na^+,K^+-ATPase, 0.2 μg/well; *Torpedo* electroplax organ microsomes, 25 μg; *Xenopus* kidney microsomes, 40 μg; chicken kidney microsomes, 40 μg; rat kidney, skeletal muscle, and brain microsomes sample contained 20 μg; purified pig gastric H^+,K^+-ATPase, 25 μg; and the purified rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase sample contained 5 μg.

ples except the *Torpedo* electroplax organ sample (not shown). This established that the variations in M8-P1-A3 binding were not due to insufficient levels of any of the tested proteins. Inspection of the known sequences for these proteins, as shown in Table 1, shows that for the region encompassed by the synthetic peptide, the eight-residue sequence VMKGAPER is identical for all of the ATPases tested, except for the rabbit skeletal muscle SR Ca^{2+}-ATPase. This protein has the Met 514 (equivalent to M 500 in the Na^+ pump) deleted and an Arg 520 → Gly substitution. Clearly, the residues VMKGAPER cannot play a significant role in forming the epitope as recognized by the M8-P1-A3 in Western blot analysis.

Analysis of the three remaining amino acids contained in the synthetic peptide (HLL) reveals sequence differences between the α-subunits, which could generate some of the observed heterogeneity in antibody binding. The *Xenopus* and *Torpedo* proteins both react poorly with the M8-P1-A3 and they both have Tyr replacing the lamb protein's His 497, whereas *Xenopus* also has the adjacent Leu 497 replaced by Ile. Thus it appears that the HLL sequences are part of the epitope. The chicken α1 sequence is even more revealing. Over the entire 18-amino acid sequence region selected for inspection (which extends beyond the 11 residues of the synthetic peptide), the only change is a Pro 494 → Ser change, which occurs two amino acids beyond the NH_2-terminus end of the synthesized peptide. Similarly, the first substitution that occurs in the rat α1 subunit is an Arg 495 → Lys change adjacent to the NH_2-terminus of the peptide. Together these results suggested that amino acids PRHLL must be part of the epitope and that the synthetic peptide contains only a portion of the epitope. They also suggested that the Pro and Arg sequences each play an important role in generating the epitope, but that a single residue change in the epitope can be partially tolerated. The *Torpedo* electroplax protein has two alterations in this sequence and is weakly reactive, whereas *Xenopus* has three substitutions (SRYIL versus PRHLL) and no binding is detected. The lamb, pig, and dog kidney Na^+,K^+-ATPases, and the pig and rat gastric H^+,K^+-ATPases, all retain the PRHL(L) sequence and are strongly recognized by M8-P1-A3.

Next, because the rat isoforms show considerable variation in the region immediately adjacent to the NH_2-terminal side of the Pro 494, Western blot analyses were conducted using three rat tissues in order to test the possibility that additional amino acids might be involved in the antibody's epitope. Adult rat kidney was used because it contains only the α1 isoform, whereas skeletal muscle contains predominately α2, and brain tissue contains all three isoforms (Sweadner, 1989). Figure 2 shows that M8-P1-A3 clearly reacts with the kidney enzyme (0.23 μg) and weakly with brain but not with skeletal muscle microsomal fractions that were matched with equivalent amounts of ouabain-inhibitable Na^+,K^+-ATPase activity (rather than total protein). By using three additional monoclonal antibodies, which have been shown to be isoform specific (McK1, α1; McB2, α2 and M7-PB-E9, α3 [Sweadner, 1989; Abbott & Ball, 1993]), as well as M8-P1-A3, we were able to confirm the isoform levels and distribution pattern while affirming that M8-P1-A3 only recognizes rat α1. Because rat α1 has the same Glu 494 as lamb α1, but the nonreactive rat α2 and α3 proteins have Gln and Asp substitutions, respectively, at this site, these data suggested that the epitope could include the following sequences, 494-EPRHLL. This would mean that the epitope

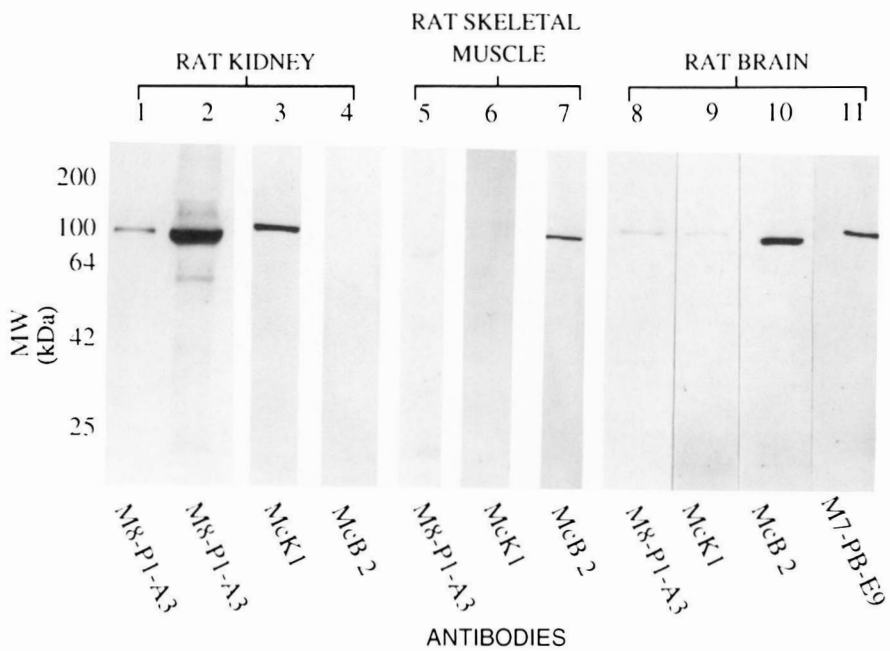

Fig. 2. Determination of M8-P1-A3 binding to rat Na^+,K^+-ATPase isoforms. Western blot analysis of M8-P1-A3 (100 nM) binding to rat kidney, skeletal muscle, and brain microsome samples is shown in lanes 1 and 2, lane 5, and lane 8, respectively. The binding of the rat α1-specific monoclonal antibody McK1, the α2-specific antibody McB2 (cell culture media diluted 1:75 and 1:40, respectively) and the α3-specific M7-PB-E9 (100 nM) is also presented. The concentrations of the microsome samples present in gel lanes were as follows: Lane 1, 3 μg; 2, 50 μg; 3, 3 μg; 4, 50 μg; 5, 20 μg; 6, 20 μg; 7, 2 μg; 8, 20 μg; 9, 20 μg; 10, 20 μg; and 11, 2 μg.

actually extends three amino acids beyond the sequences present in the synthetic peptide used originally to identify the epitope region.

Epitope identification through the isolation of M8-P1-A3-specific, epitope-containing bacteriophage

In order to confirm our deductive work with the species-specificity mapping presented above, the M13 phage (M13LP67) random peptide library designed by Devlin et al. (1990), which contains a 15-amino acid random peptide sequence insert in the pIII protein (encoded in gene III), was used to isolate a population of phage capable of being bound by the antibody. After several rounds of selection and enrichment steps, done using petri dish-absorbed antibody, to select for binding phage, individual phage isolates were obtained and their insert regions of gene III were sequenced. Seventeen independently derived clones gave 14 different sequences. As shown in Table 2, two colonies were found to have the identical 15-amino acid sequence insert, including the five-residue group of PRHLL, which was a perfect five-residue match to the lamb α1 and included five of the six amino acids predicted from the species-specificity mapping (EPRHLL) work.

Table 2. *The bacteriophage "epitope library" sequences obtained by selection with monoclonal antibody M8-P1-A3*[a]

Source	Sequence	Number of clones
Sheep α1 NKA	GE **PRHLL** VMKGAPER	
Synthetic peptide	HLL VMKGAPER	
Isolate phage,	MSRNFSGTT **PRHLL** L	2
insert sequences	SVHQLNA **PRSLL** FNH	1
	LP **PRNLL** YGNSRLDW	1
	YRPISNM **PRQLL** MRL	1
	MDT **PRHLM** AYLITQR	2
	LMNS **PRQLM** TRKMSH	1
	TIPSAKS **PRSLF** VTH	1
	STSP **PRSLL** GSLDRT	1
	LTHHPHY **PRHLM** GSR	1
	LPPSEAST **PRRLL** HQ	1
	PRSLL SPSDSLRFIT	1
	PRNLF LPTAHPFST	1
	TEFITRE **ARHLL** LIP	1
	FQEPKSE **QRHLF** YKW	2
Consensus sequence	PRHLL	

[a] The M8-P1-A3 binding phage were separated from the initial total pool of phage by the selection steps described in the Materials and methods. The amino acid sequences of the 15-amino acid inserts that were present in 17 different phage isolates are presented and the deduced sequence among these phage was PRHLL. This sequence is contained in the lamb kidney α1. The observed degeneracies in the sequences that bound to M8-P1-A3 were consistent with Western blot analysis data, which showed monoclonal antibody binding to the chicken and rat 1 proteins despite their respective Pro 494 → Ser and Arg 495 → Lys changes, and pig gastric H^+,K^+-ATPase with the deletion of Leu 498.

Analysis of all of the insert sequences further confirmed these results with the consensus sequence indeed being PRHLL, whereas a match of at least 3 of the 5 amino acids was necessary to produce binding phage. In terms of residue frequencies, Pro was present in 12/14 isolates, Arg = 14/14, His = 7/14, Leu = 14/14, and Leu = 10/14. Therefore, the essential epitope configuration was PRhLl. There was also just a suggestion of the site being extended two more residues with two clones having the appropriate Glu (E) and four clones Leu or Val (L/V) for the adjoining amino acid residues on the respective NH_2- and -COOH terminal sides of this sequence. There was, however, no apparent pattern for the location in the 15-amino acid stretch that the 3–5 matching residues began. The degeneracy observed in the amino acid sequences of the binding phage was consistent with the observations that α proteins, such as the chicken and rat α1 kidney proteins, with their respective Pro 494 → Ser and Arg 495 → Lys changes, and the pig gastric H^+,K^+-ATPase, with its Leu 498 deletion, were still recognized by the antibody.

Interestingly, no phage were isolated that bound while containing only the HLL sequence present in the synthetic peptide. Because M8-P1-A3 binding was observed with the HLLVMKGAPER–BSA conjugate but not the –KLH conjugate, it seems reasonable to speculate that either a Pro or Arg on the carrier protein was in a position close enough to the coupled peptide to generate a more complete epitope, or that the coupling reagent used with BSA (EDAC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide) is able to substitute for a portion of the antibody-binding site, whereas *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS), used for KLH, is not. These data clearly demonstrate the capability of the epitope library system to identify simple continuous epitopes and to delineate the fine specificity requirements of antibody binding. This technique, although inspired by the "mimotope" short random peptide microsynthesis procedure developed by Geysen et al. (1986), appears to have many advantages in terms of its convenience and the information generated. It should prove to be a powerful tool for the study of protein–ligand interactions (Cesareni, 1992). Our results also suggest that in favorable situations, species-specificity mapping can also yield very detailed information. Together these two methods have enabled us to achieve the first definitive identification of a holoenzyme epitope site on the Na^+,K^+-ATPase.

Epitope accessibility and its functional role

In terms of understanding both the structure and function of the enzyme, it is interesting how closely M8-P1-A3 binds to Lys 501, which is labeled covalently by FITC, without disturbing either the probe's fluorescent properties or its spectral responses to ligand binding to the enzyme. Bound antibody also does not affect the enzyme's

ATPase or *p*-nitrophenylphosphatase activity at saturating ligand concentrations. This contrasts with the results obtained with three inhibitory monoclonal antibodies investigated in this laboratory (Ball, 1986; Friedman & Ball, 1989; Abbott & Ball, 1992). Our studies (Ball & Friedman, 1987) have shown that the M8-P1-A3 epitope does undergo Mg^{2+}-, Mg^{2+}-ATP-, and Mg^{2+} + Pi-dependent conformational changes that alter the antibody's binding affinity, but these changes are not sufficient to displace already bound antibody. This suggests that the epitope region is exposed but does not play a critical role in either ATP binding or catalytic turnover.

Alternatively, as mentioned earlier, according to the ATPase composite model of the nucleotide-binding site proposed by Taylor and Green (1989), the Pro-Arg sequences of PRHLL should be part of the terminating turn of an α-helix; with the HLL(VM) residues forming a β-sheet; the KGA residues, an ATP-binding loop; followed by an α-helix (PERILDR). Garnier analysis (Garnier et al., 1978) of the lamb kidney $\alpha 1$ sequences only also suggests essentially the same secondary structure arrangement for this enzyme. This would mean that most of the M8-P1-A3 epitope is internalized within the cleft, which forms a portion of the adenine-binding pocket. The surface exposure of residues 494–498 is inconsistent with the model. Further, it has been suggested that our previous studies indicate that M8-P1-A3 binds only a 1% denatured fraction of the purified enzyme (Xu & Kyte, 1989). The question then is how convincing is the evidence that M8-P1-A3 binds active enzyme?

To answer this question, we can compare its binding properties with those of three other monoclonal antibodies generated against the holoenzyme, which are inhibitory and clearly do bind to active enzyme: M10-P5-C11, 9-A5, and M7-PB-E9. The first two antibodies recognize active enzyme but not Western-blotted α, whereas M7-PB-E9 binds both native and denatured α. All four antibodies were identified originally through their binding to plastic microtiter plate-adsorbed enzyme using an enzyme-linked immunoadsorbent assay (ELISA). In these assays M8-P1-A3, M10-P5-C11, and M7-PB-E9 have nearly identical (2.5, 3.5, and 2.9 nM, respectively) concentration values for half-maximal binding (Ball & Lane, 1986; Abbott & Ball, 1992), whereas 9-A5 (raised to the rat kidney enzyme) does not bind our plate-adsorbed lamb enzyme. As far as the level of antibody binding in this assay, M7-PB-E9 and M8-P1-A3 both bind at about 40% higher levels than M10-P5-C11.

When the enzyme is adsorbed to nitrocellulose sheets for analogous ELISA-type dot blots, the enzyme appears to be more native than the plate-adsorbed enzyme because the inhibitory antibody 9-A5 now also binds. In dot blots at low antigen levels and 10 nM antibody concentrations, both M8-P1-A3 (<50%) and M7-PB-E9 show lower levels of binding than do M10-P5-C11 and 9-A5. Consistent with this reflecting only a difference in relative affinities, when the antibody concentrations are raised to 100 nM, this differential in the level of binding is largely abolished, whereas densimetric quantitation of the blots shows, that at 1,000 nM, levels M8-P1-A3 binding is equal to that of M10-P5-C11 and 9-A5.

Finally, when "in solution" immunoprecipitation studies were done to determine the ratio of enzyme (a particulate suspension) needed to bind 50% of a limiting fixed amount of antibody, we found that M8-P1-A3 in Tris buffer (plus or minus Na^+ or K^+) requires only a fourfold higher level of enzyme than that required to achieve the same level of enzyme-antibody complex as that for M10-P5-C11 (Ting-Beall et al., unpubl.). Furthermore, we have also adapted an enzyme "capture assay," in which we determine the ability of plate-adsorbed antibody to bind and immobilize enzyme from solution. In this work, we similarly found that M8-P1-A3 requires only fourfold more antibody than M10-P5-C11 to achieve a half-maximal level of bound enzyme, with the total amount binding being at least 85% of that bound maximally by either M10-P5-C11 or 9-A5. Clearly, M8-P1-A3 does not have binding properties distinct from those of the other antibodies. It binds to the purified enzyme using several different procedures, including ones where the enzyme is not denatured, and it can bind at levels similar to that of antibodies having high affinity for active enzyme. It does this in assays that require essentially an irreversible binding.

Consistent with our proposal that the PRHLL sequence is surface exposed are the results of Tunwell et al. (1991b) that identify the five-amino acid sequence, VGNKM, as the epitope for monoclonal antibody Y/1F4, which is directed against the rabbit skeletal SR Ca^{2+},Mg^{2+}-ATPase. This antibody also recognizes both native and Western-blotted enzyme and causes a modest (~17%) inhibition of activity (Tunwell et al., 1991a). This sequence stretch matches exactly the placement of the PRHLL residues in the Na^+ pump save for a one-residue shift toward the NH_2-terminus. In addition, in a somewhat less rigorous manner, Van Uem et al. (1991) have identified the nonidentical, six-residue region adjacent to Pro 494 of the pig gastric H^+,K^+-ATPase (ledprd*P* vs. NANAGE*P* for Na^+,K^+-ATPase) as the exposed epitope for the inhibitory monoclonal 5-B6. Finally, Xu and Kyte (1989) have shown that polyclonal antibodies raised to the -GAPER sequence residing on the -COOH terminal side of Lys 501 also bind native enzyme and inhibit activity. This suggests that the entire 20-amino acid region of 488–506 is largely accessible to antibody binding, which would be at odds with current structural models.

Whether Lys 501 is essential in forming the nucleotide-binding domain, indeed, remains in question given the relatively minor effects on activity that have thus far been reported upon mutation of this site in both the Ca^{2+} and Na^+ pumps (Maruyama et al., 1989; Ohtsubo et al., 1990). Further, its accessibility to antibody binding makes it seem less likely to participate directly in either FITC or

ATP binding, but the basic residue of Lys 501 may be oriented uniquely. Its solvent accessiblity in terms of its reactivity with acetic anhydride is reduced by the $E_1 \rightarrow E_2$-P transition and by the presence of ATP (Xu & Kyte, 1989). Also, recent spectroscopic evidence from our lab (Abbott et al., 1991) has shown that specifically bound FITC resides in a restricted hydrophobic region, which would suggest that the amine group of Lys 501 faces toward the nucleotide-binding site and is in close proximity to bound ATP or FITC.

It should also be acknowledged that it is not known to what extent particular functional domains of closely related proteins are structurally identical. It is interesting to note that the epitope sequences of the rat $\alpha 1$ and pig/rat gastric H^+,K^+-ATPase proteins are similar enough to be recognized by M8-P1-A3 when subjected to Western blot analysis, but these enzymes do not react with the antibody when they are in the more native form used for either ELISA (microtiter plate) or dot blot (nitrocellulose paper) adsorption assays. This suggests that there are adjacent residues that help generate the entire epitope and that these differ among the different E_1E_2 enzymes or species forms of Na^+,K^+-ATPase, or that the amino acid substitutions that occur in the identified five-residue epitope region produce secondary or tertiary structural differences from the lamb enzyme that greatly reduce antibody affinity. M8-P1-A3 is sensitive to these alterations in its epitope site despite the fact that all of the E_1E_2 ATPases seem to have ATP sites with similar ATP affinities, to form the same E-P intermediates, and are labeled by FITC at the equivalent Lys 501. It is apparent that protein folding is a complex process and there may be alternative ways for similar proteins to achieve the same functional responses. Monoclonal antibodies such as M8-P1-A3 should continue to be useful tools in identifying exposed protein sites and probing the differences in folding between closely related enzymes.

Materials and methods

Enzyme and tissue preparation

Na^+,K^+-ATPase was purified from lamb kidney medulla according to the method of Lane et al. (1979). This preparation had an initial activity of approx. 1,000 μmol Pi/mg protein/h. Using essentially the same procedures, somewhat less-active preparations were also obtained from dog, pig, and rat kidneys. Microsomal preparations from chicken, rat, and *Xenopus* kidney, rat brain, gastric mucosal cells, and skeletal muscle, and *Torpedo* electroplax organ were prepared by differential centrifugation of homogenized tissue. Frozen or fresh tissues were homogenized in approximately nine volumes of 0.03 M imidazole, 2 mM EDTA, 0.25 M sucrose, pH 7.2, and centrifuged at 10,000 × *g*, for 45 min. The supernatant fraction was then centrifuged at 30,000 × *g* for 45 min and the pellet collected. Purified rabbit skeletal muscle SR Ca^{2+}, Mg^{2+}-ATPase was provided by Dr. Evangeline Kranias (University of Cincinnati), and purified pig gastric H^+,K^+-ATPase was provided by Dr. John Cuppoletti (University of Cincinnati).

Antibody isolation: ELISA, dot blot, and Western blot analysis

Monoclonal antibodies M8-P1-A3, M7-PB-E9, and M12-P4-E8 were raised against the lamb kidney Na^+,K^+-ATPase and are α-subunit specific. They were generated and purified chromatographically from mouse ascites fluid by procedures reported previously by Ball et al. (1982, 1988). The rabbit (New Zealand, white) polyclonal antiserum designated 37292 was raised against purified lamb kidney α-subunit according to Ball and Schwartz (1982). The anti-rat $\alpha 1$ antibody McK1 and anti-rat $\alpha 2$ antibody McB2 were supplied by Dr. Kathleen Sweadner (Harvard Medical School). Binding of antibody to the enzyme preparations was measured using three methods: (1) An indirect solid-surface absorption binding assay (ELISA), which has been standardized previously (Ball et al., 1982) and in which the antigens are adsorbed to plastic microtiter plate wells (Cooke flexible plates) and then exposed to the monoclonal antibodies. A reversed "capture assay" was also used. In this procedure the monoclonal antibody is adsorbed to the plates and then the antigen (Na^+,K^+-ATPase, with carrier BSA) is allowed to bind to the monoclonal antibody while a rabbit polyclonal antisera (37292) is used to quantitate the level of captured enzyme. An alkaline phosphatase–streptavidin conjugate was used to detect the binding of biotinylated sheep anti-mouse IgG or biotinylated sheep anti-rabbit IgG, immunoglobulin (F(ab)'2). (2) An immunoblot, or dot blot, assay in which the antigens were adsorbed to nitrocellulose paper and then the paper was exposed to antibody. Bound mouse antibody was then detected using a biotinylated sheep anti-mouse IgG secondary antibody and a peroxidase–streptavidin conjugate. (3) Finally, Western blot analyses were accomplished by the electrophoretic transfer of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% or 10% Laemmli gels [Laemmli, 1970]) resolved proteins onto nitrocellulose sheets (Towbin et al., 1979). These sheets were then exposed to the various specific antibodies, and either horseradish peroxidase or alkaline phosphatase conjugated to appropriate anti-mouse or anti-rabbit secondary antibodies was used to detect the antigen–antibody complexes.

Epitope library—Selection of antibody-specific phage

The filamentous bacteriophage library consists of a population of M13mp19 phage each with a random 15-amino acid sequence region inserted into the gene III protein,

pIII. This library, which has been described by Devlin et al. (1990), was obtained from the Chiron Corporation (Emeryville, California). We then used a selection procedure based on that described by Devlin et al. (1990) and Scott and Smith (1990) to isolate phage that contained random peptide sequences recognized by antibody M8-P1-A3. In this procedure, polystyrene petri dishes (60 × 15 mm) were incubated with 4 mL of M8-P1-A3 (100 μg/mL) in 1 mM Tris-EGTA, pH 7.4, overnight at room temperature in an air-tight humidity box. Because saturation binding of the antibody to these plastic plates was demonstrated to occur at about 6 μg/mL, the initial concentration of antibody used was in excess. The antibody solution was then removed and the plate was incubated with 10 mL of blocking buffer (Tris-buffered saline [TBS], 3% BSA, and 3% normal goat serum) for 1 h with shaking. The dish was then washed three times with a TBS/0.05% Tween-20 buffer. The treated plate was then incubated for 2 h with a 100-μL aliquot of the phage (5×10^{10} phage) diluted into 2 mL of the blocking buffer. The solution was then removed and the plate washed 10 times with a TBS/0.5% Tween 20 buffer over a 2-h period. The phage that remained bound to the plate were eluted using 1 mL of sterile elution buffer (1 mg/mL BSA and 0.1 N HCl adjusted to pH 2.2 with glycine), which was then neutralized with 60 μL of 2 M Tris as described by Parmley and Smith (1988). The eluted phage (4×10^3 phage) were amplified by plating the phage along with log-phase growing *Escherichia coli* (XL-1 Blue, Stratagene) in top-agarose solid media at a density of about 50,000 plaques/100-cm^2 plate, overnight, and then adding 5 mL of suspension medium buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.01% gelatin, and 0.05 M Tris-HCl, pH 7.5) to the agar plates and letting them incubate at 4 °C for an additional 6–12 h with occasional shaking. The suspension medium solution was removed from the plates, and the residual bacteria were removed from the phage-containing solution by centrifugation at 10,000 × *g* for 10 min (Zinder & Boeke, 1982). A second-selection procedure was then done in which a sample of 6×10^9 recovered phage were exposed to a dish treated with a reduced concentration of antibody (5 μg/mL). About 1% of the added phage were recovered from this plate subsequent to the binding, washing, and elution steps. In comparison, a petri dish coated with 100 μg/mL of the monoclonal antibody gave a recovery of 1.5%. The phage eluted from the 5-μg/mL antibody-treated plate were again amplified by growth in the top-agar medium and a third selection done. In this selection process, the phage were first bound to and eluted from a petri dish coated with 10 μg/mL of antibody. The eluted phage were exposed for 1 h to a petri dish coated with blocking buffer only, and the unbound phage were collected. These phage were allowed to bind overnight in a third petri dish that was precoated with 10 μg/mL of antibody, and the bound phage were eluted (0.6% of the 6×10^9 phage added). The recovered phage were grown up, following appropriate dilution into the solid agar medium containing *E. coli*, on petri plates at a density of about 40–50 phage/plate; 17 individual clear plaques representing individual phage colonies were isolated and amplified by growth in liquid media for sequencing.

DNA isolation and sequencing

To sequence the random peptide insert regions of the phage, single-stranded DNA templates were obtained by polyethylene glycol (PEG) precipitation of the phage present in the liquid culture samples, followed by phenol extraction and ethanol precipitation (Ausubel et al., 1989) of the DNA. After quantitation of the isolated DNA samples, they were sequenced using the Sequenase 2.0 USB kit, and ^{35}S-dATP according to the manufacturer's instructions. The template primer used had the sequence ACAGACAGCCCTCATAGTTAGCG, which hybridizes with the nucleotide resides starting 109 bases from the site of the random peptide library insert.

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